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miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles

Mehmet Fatih Bolukbasi[1], Arda Mizrak[1], Gokhan Baris Ozdener[1], Sibylle Madlener[2], Thomas Ströbel[3], Erdogan Pekcan Erkan[2], Jian-Bing Fan[4], Xandra O Breakefield[1] and Okay Saydam[2]

Despite intensive studies, the molecular mechanisms by which the genetic materials are uploaded into microvesicles (MVs) are still unknown. This is the first study describing a zipcode-like 25 nucleotide (nt) sequence in the 3′-untranslated region (3′UTR) of mRNAs, with variants of this sequence present in many mRNAs enriched in MVs, as compared to their glioblastoma cells of origin. When this sequence was incorporated into the 3′UTR of a reporter message and expressed in a different cell type, it led to enrichment of the reporter mRNA in MVs. Critical features of this sequence are both a CUGCC core presented on a stem-loop structure and a miRNA-binding site, with increased levels of the corresponding miRNA in cells further increasing levels of mRNAs in MVs.
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Introduction

Membrane-derived microvesicles (MVs) include a range of extracellular vesicles, including exosomes, microparticles, and shed MVs secreted by many cell types under both normal physiological and pathological conditions.[1] As intercellular communication tools, MVs have been reported to have roles in a wide range of cellular functions: immunological modulation, coagulation, and tumor progression, including angiogenesis and metastasis.[2,3] Additionally, they have been reported to serve as vehicles for transferring cargo (mRNA, miRNA, noncoding RNAs, proteins, and oncogenes) between cells.[4–8] The mRNA content in MVs open new research opportunities from cancer diagnostics to gene therapy applications.[9–12] Despite, the intensive research in analyzing the RNA content of MVs, it remains unclear how RNAs are directed to MVs.

Mechanistically, cis-acting regulatory sequences and trans-acting proteins are considered as the main driving forces of mRNA localization within cells and have an important role in post-transcriptional regulation. Such sequences, also known as zipcodes, are typically found in the 3′-untranslated regions (3′UTRs) of mRNA transcripts and mediate binding of a ribonuclear protein complex to the mRNA which temporarily blocks its translation and mediates movement via the cytoskeleton to a cellular location where it releases the mRNA and translation commences.[13,14]

In addition to zipcodes, microRNAs (miRNAs) also have a critical role in post-transcriptional regulation of mRNAs. miRNAs are small, noncoding, single-stranded RNA molecules (~21–23-nucleotides (nt) long) that regulate levels of gene expression in many organisms.[15] miRNAs mediate post-transcriptional regulation in three ways, by mRNA degradation, mRNA destabilization via deadenylation, and translational repression.[16] In addition to negative regulation, miRNAs have also been reported to function in the activation of translation in some cases.[17] Although zipcodes and miRNA target sequences are both found in the 3′UTRs of the mRNA transcripts, there has been no report of cooperation between these two regulatory mechanisms in mRNA fate.

In this study, we investigated whether there is a sequence in the 3′UTR of a subset of mRNAs that are enriched in MVs which may act like a zipcode to target them into MVs. Our multiple sequence alignment analysis comparing the cellular transcriptome of human primary glioblastoma multiforme (GBM) cells with the transcriptome of MVs derived from them,[5] revealed a stem loop-forming sequence of 25 nt, variations of which were present in some of the most MV-enriched mRNAs. This putative zipcode sequence contained a single miRNA-binding site for miR-1289, as well as a CTGCC core sequence. Mutational analysis showed that these sequences cooperated in enrichment of a reporter mRNA in MVs and that upregulation of miR-1289 levels in cells further enhanced MV enrichment.

Results

Microarrays of the mRNAs isolated from GBM-derived MV reveals a zipcode-like sequence. To test the hypothesis that there is a sequence in the 3′UTR of mRNAs that are enriched in MVs which serves as a zipcode to target them to MVs, we analyzed microarray studies of the RNA content of two human primary GBM cells and two primary

The first two authors contributed equally to this study.

[1]Department of Neurology and Radiology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA; [2]Molecular Neuro-Oncology Research Unit, Division of Oncology, Department of Pediatrics, Medical University of Vienna, Vienna, Austria; [3]Institute of Neurology, Medical University of Vienna, Vienna, Austria; [4]Illumina, Inc., San Diego, CA, USA.
Correspondence: Okay Saydam, Molecular Neuro-Oncology Research Unit, Division of Oncology, Department of Pediatrics, Medical University of Vienna, Vienna 1090, Austria.
E-mail: okay.saydam@meduniwien.ac.at
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melanomas, and MVs derived from them. We focused on the 20 mRNAs most enriched in MVs as compared to cells of origin (**Supplementary Table S1**) and performed extensive analysis of 3′UTR sequences of these mRNAs using a multiple alignment program (Clustal W2-http://www.clustal.org/). Among those 20 transcripts, NM_003614.1 (mRNA GalR3) gave the highest pairwise alignment score within the list. Hence, we decided to evaluate the 25-nt sequence of this transcript with the highest consensus among these enriched mRNAs (**Figure 1**). The similarities between this 25-nt sequence and the 3′UTR sequences of the top 20 MV-enriched transcripts was later deciphered with BLAST pairwise alignment. Within the conserved regions, 25 nt are picked for further ClustalW alignment which resulted in a 5-nt core sequence "CTGCC" (or variations CTGC, CTCCC, CGCCC, TGCC) shown in a red rectangle in 11 of these 20 MV-enriched mRNAs (**Figure 2**).

Zipcode fused to the EGFP mRNA is functional. To examine the possible effect of this 25-nt sequence on incorporation of mRNA molecules into MVs, we incorporated this sequence into the pEGFP-N1 plasmid (Clontech, Mountain View, CA) by removing most of the original 3′UTR sequence of the enhanced green fluorescent protein (EGFP) mRNA. The resulting plasmid expresses EGFP mRNA fused to this potential zipcode, followed by a polyA addition site and referred to here as pEGFP-N1-3UTR-25nt. Intact pEGFP-N1 plasmid was used as a control. We first tested whether the new EGFP mRNA was stable without its original 3′UTR. Human embryonic kidney-293T (HEK-293T) cells were transfected with either wild-type EGFP-expressing plasmid pEGFP-N1 or pEGFP-N1-3UTR-25nt. Twenty-four hours later, total RNA was isolated from cells and quantitative reverse transcription qRT-PCR was performed for EGFP mRNA. As shown in **Figure 3a**, we observed a similar increased accumulation of the EGFP mRNA 24 hours after transfection with both pEGFP-N1 and pEGFP-N1-3UTR-25nt constructs. To evaluate whether the EGFP mRNA fused to the "25-nt sequence" was efficiently translated into EGFP protein, we also examined these transfected cells by fluorescence microscopy. As shown in **Figure 3b,c**, similar EGFP protein expression levels were observed for pEGFP-N1 (lanes 1 and 2) and pEGFP-N1-3UTR-25nt (lanes 3 and 4) over time suggesting that 25-nt long sequence is compatible with stability and efficient translation.

We next tested whether this 25-nt sequence might be sufficient for enrichment of EGFP mRNA's in MVs. We transfected HEK-293T cells either with control plasmid or pEGFP-N1-3UTR-25nt and 72 hours later, we harvested MVs and cells and performed qRT-PCR for EGFP and GAPDH mRNAs. We observed that this sequence increased the amount of EGFP mRNA in MVs by about twofold, as compared with the original construct (**Figure 4a**). We also tested whether the presence of the 5-nt core sequence "CTGCC" is required for enrichment. When we mutated the 5-nt core sequence in two different ways, namely MT1 and MT2 (**Figure 4b**, **Supplementary Figure S1** and **S2**), mRNA enrichment inside the MVs was inhibited (**Figure 4c**). To avoid any plasmid DNA contamination during MV isolation, samples were treated with DNase before qRT-PCR, confirming that the data observed is due to mRNA content in MVs (**Supplementary Figure S3a,b**). Moreover, to investigate the origin of the signal received by qPCR, we performed the following treatments: *DNase*I on the outside of MVs and content of MVs, and RNase treatment before and after RNA isolation from MVs. As shown in **Supplementary Figure S3c**, *DNase*I treatment did not prevent amplification of the PCR product, whereas after RNase treatment of the contents of MVs, no signal was observed suggesting that the signal observed in our experimental conditions came from mRNAs isolated from inside of the MVs not from any plasmid DNA contamination during MVs isolation or mRNAs attachment to the MVs.

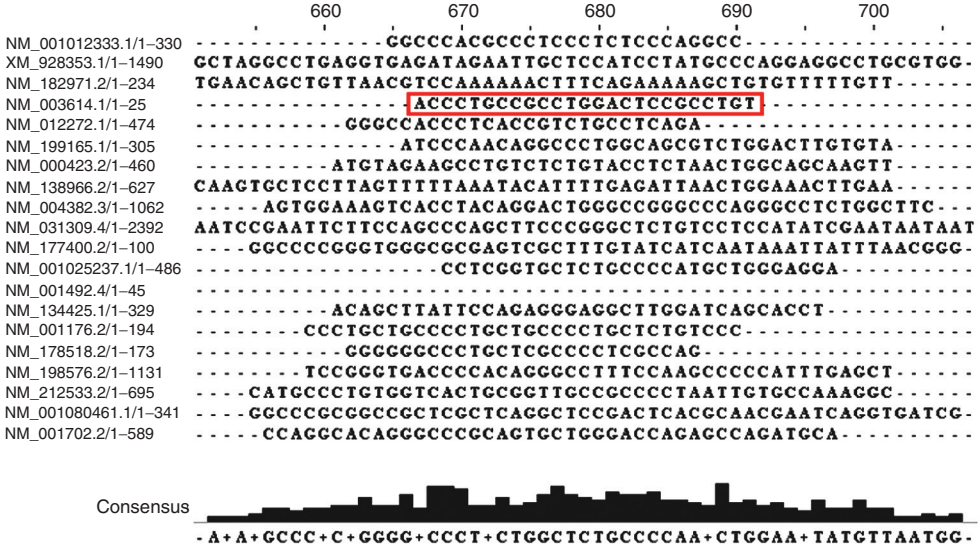

Figure 1 Alignment of the 3′UTRs of top 20 enriched transcript. 3′UTR sequences of the top 20 enriched transcripts were obtained from National Center for Biotechnology Information (NCBI) and Ensemble databases. The sequences were aligned with ClustalW a multiple sequence alignment program. The 25-nt sequence we used in this study and refer as the zipcode is shown in the red rectangle. nt, nucleotide; UTR, untranslated region.

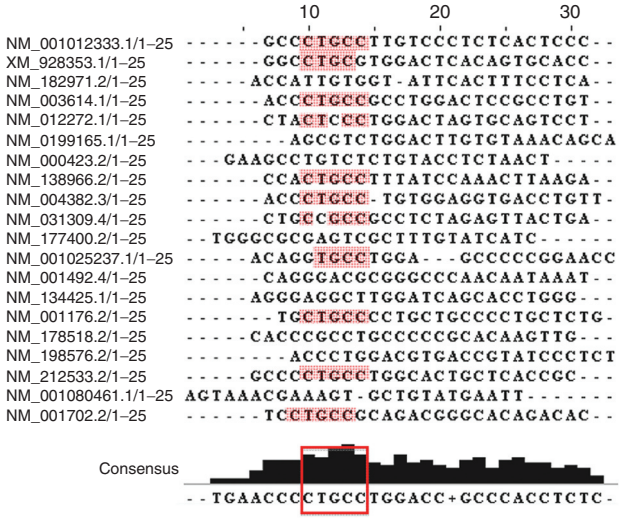

Figure 2 Deep alignment of the 3′UTR of the top 20 enriched transcripts. The similarities between the 25-nt sequence and the 3′UTR sequences of top 20 transcripts were deciphered with BLAST pairwise alignment. Within the conserved regions, a 25-nt sequence was picked for further ClustalW alignment. The 5-nt core sequence "CTGCC" is shown in the red rectangles. nt, nucleotide; UTR, untranslated region.

miR-1289 directly binds to 25-nt zipcode and, in part, has a cooperative role in the enrichment of mRNAs within MVs. We further investigated the role of the miRNA-binding sequence in the 3′UTR region of the reporter mRNA in transfer of the EGFP-25nt mRNA into MVs. Based on the facts that miRNAs are a family of 19–24 nt noncoding RNAs that inhibit the expression of target mRNAs by binding to complementary sequences in the UTRs of mRNAs (typically the 3′UTR) and repressing translation and/or cleaving the mRNA, we hypothesized that an miRNA might be involved in this MV targeting mechanism. To test this hypothesis, we performed miRNA database search (http://www.mirbase.org/) within the 25-nt putative zipcode sequence and found a potential binding site for miR-1289 (**Figure 5a**). Strikingly, when we co-transfected cells with the EGFP-25nt cassette and pre-miR-1289, MV enrichment of the mRNA increased to sixfold, whereas when we silenced endogenous miR-1289 by transfection with its anti-miR-1289 sequence, we observed inhibition of EGFP-25nt mRNA enrichment within MVs (**Figure 5b**). In these experimental conditions, after transfection of pre-miR-1289, miR-1289 levels were increased ~100-fold in cells and 40-fold in MVs as assessed by qRT-PCR, while endogenous miR-1289 levels in cells were below the detection level of RT-PCR after inhibition of miR-1289 by anti-miR-1289 (data not show). The miR-1289-

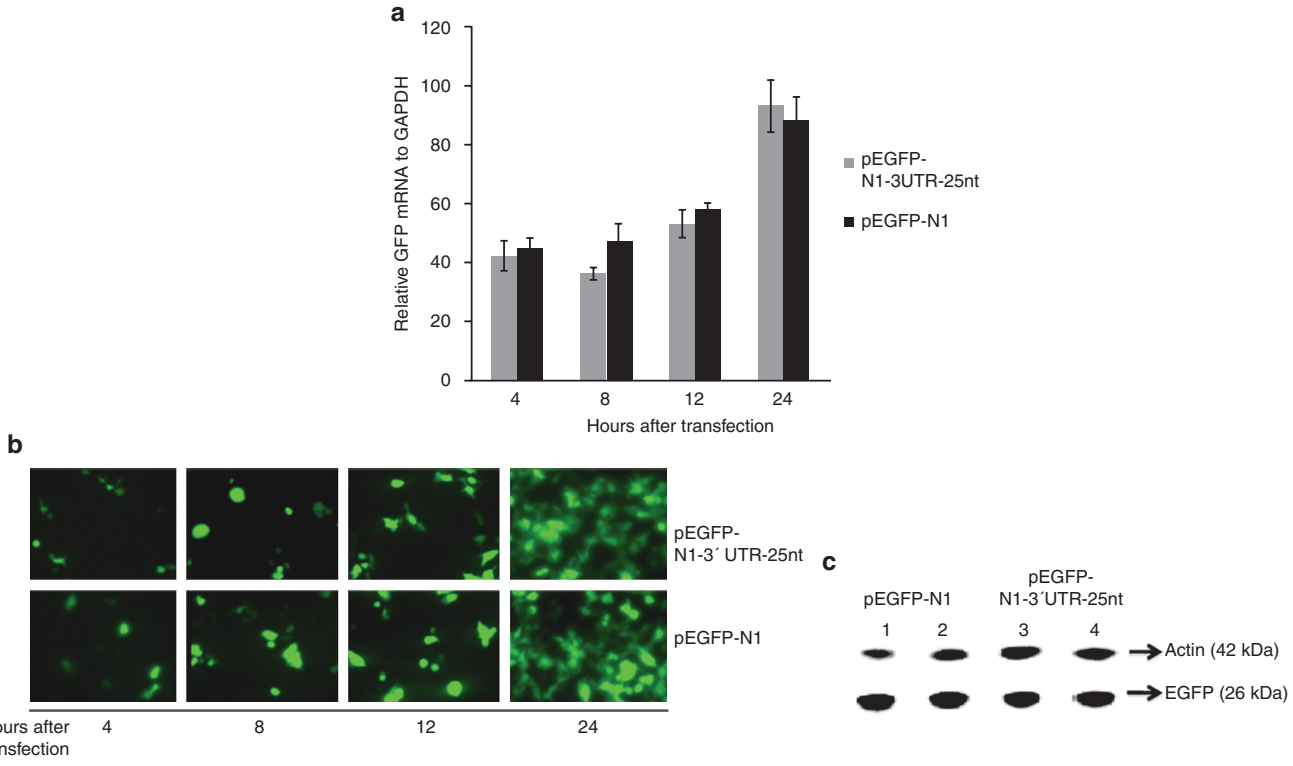

Figure 3 The EGFP mRNA with the zipcode in the 3′UTR is stable and translated. HEK-293T cells were transfected either with pEGFP-N1 or pEGFP-N1-3UTR-25nt plasmids. (**a**) Total RNA was isolated at indicated time points and quantitative reverse transcription (qRT)-PCR was performed for EGFP and GAPDH mRNAs. EGFP mRNA levels were normalized to GAPDH mRNA. (**b**) Under the same experimental condition, cells were examined under fluorescence microscope for EGFP expression at the same time points (×20 magnification). (**c**) HEK-293T cells were transfected as above and western blot analysis was performed with antibodies to EGFP and β-actin. pEGFP-N1 (lanes 1 and 2) and pEGFP-N1-3UTR-25nt (lanes 3 and 4). nt, nucleotide; UTR, untranslated region.

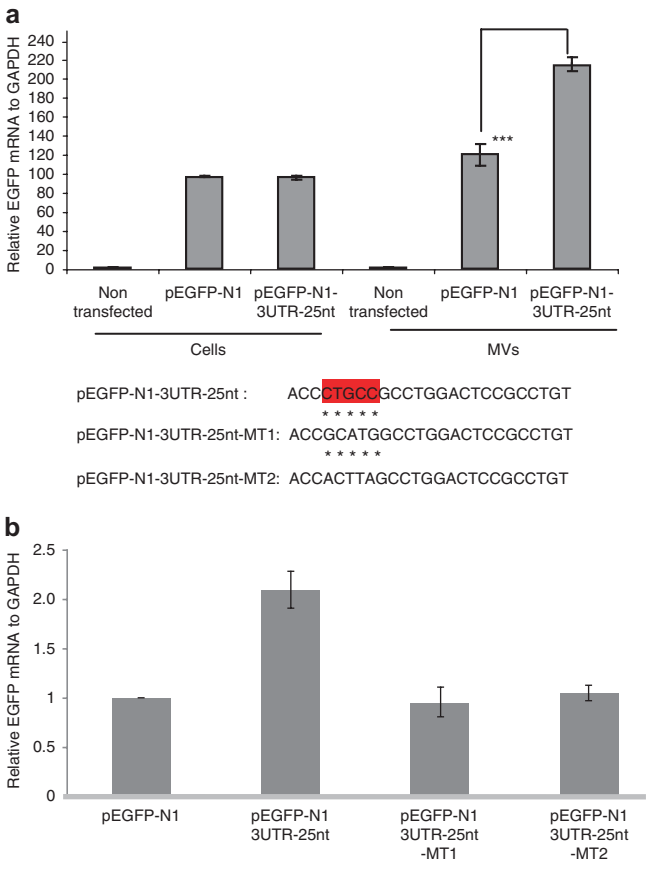

Figure 4 Functionality of zipcode in the transfer of messages into microvesicles. (**a**) HEK-293T cells were nontransfected or transfected with either wild-type EGFP-expressing plasmids, pEGFP-N1-3UTR-WT or pEGFP-N1-3UTR-25nt. Seventy-two hours later, total RNA was isolated from cells and microvesicles (MVs), and quantitative reverse transcription (qRT)-PCR were performed for EGFP and GAPDH mRNAs. The data were normalized to the level of GAPDH mRNA in each sample. GAPDH mRNA levels were similar in cells transfected with either construct, as well as in the MVs derived from them. Five independent experiments were performed in triplicate and the values are expressed as mean ± SD using Student's *t*-test; ***$P < 0.001$. (**b**) Mutated sequences in the zipcode are shown for MT1 and MT2 constructs. (**c**) Similar MV enrichment experiments were performed as in (a) using the wild-type and mutated constructs shown in (**b**).

mediated enrichment process is not due to the excessive expression of it either in HEK-293T or primary GBM cells, because miR-1289 was found to be expressed in low levels in those cells (data not shown). We further tested the role of the potential miR-1289 binding sequence in MV targeting of the mRNA by generating point mutations within the miR-1289 target sequence (**Supplementary Figure S2**; MT3 and MT4) and observed that a mutation in the miR-1289 binding site (MT4) led to significant reduction in the accumulation of the EGFP-25nt mRNAs in MVs (**Figure 5c**). The finding that the MT3 mutation did not dramatically inhibit the accumulation of the EGFP mRNA in MVs suggests that not only the miR-1289 binding site, but also the intact core sequence within the stem-loop structure are involved in miR-1289-meditated transfer of mRNAs into MVs.

To test whether miR-1289 directly binds to this 25-nt sequence, we constructed pMir-Reporter plasmids carrying either the wild-type 25-nt zipcode or a zipcode with mutations in the core-binding site of miR-1289. The same mutations were introduced into pMir-Reporter vectors as in MT4 (**Supplementary Figure S2**). Co-transfection of the wild-type pmiR-zipcode and pre-miR-1289 resulted in significantly decreased luciferase activity as compared with transfection with pre-control 1, whereas transfection with the MT4 mutated sequence did not affect activity levels (**Figure 5d**). In order to test whether miR-1289 overexpression could affect MV incorporation of endogenous mRNAs, we picked two mRNAs from **Supplementary Table S1** based on their miR-1289 binding status and performed similar experiments as in **Figure 5b** for GALR3 (one miR-1289 binding site in the 3′UTR) and MDK (no miR-1289 binding site) mRNAs and found that miR-1289 expression significantly increased GALR3 mRNA ratio (MVs/cell) by ~50%, as compared to pre control 1, whereas miR-1289 had no effect on the level of MDK mRNA in MVs (**Figure 5e**). These data suggest that miR-1289-mediated enrichment via the zipcode sequence functions for endogenous mRNAs as well.

Next, we looked for the presence of miR-1289 target sites within 3′UTRs of the top 50 enriched mRNAs, and found that 14 of these mRNAs exhibited computationally predicted target sequences in their 3′UTR (**Supplementary Table S2**). To test whether decreased uptake of the reporter mRNA with a mutant zipcode into MVs was due to increased degradation of mRNA within cells, we also performed RT-PCRs for the cellular EGFP mRNA. As shown in **Figure 6a**, there was no significant difference in levels of mutant and wild-type EGFP mRNAs in cells over a 48-hour period of time. In addition, in cells co-transfected with pre-miR-1289 and pEGFP-N1-3UTR-25nt, EGFP expression was not downregulated indicating that there was no inhibition of translation in these conditions (**Figure 6b**). qRT-PCR also showed that cellular mRNA levels of pEGFP-N1 3′UTR-25nt were not altered compared to control vector (**Figure 6c**). Taken together, our data suggested that miR-1289 does not inhibit translation of the EGFP-N1 3′UTR-25nt mRNA, but rather may provide a means of novel posttranscriptional gene silencing mechanism by mediating the transfer of this mRNA into MVs.

Our MV mRNA enrichment assay findings suggest that the presence of both the core "CTGCC" region and the miR-1289 binding site promote targeting of mRNA into MVs. To determine the extent to which these two elements are present within enriched and reduced sets of mRNAs in MVs, we searched the 3′UTR sequences of the top 50 enriched and 50 reduced mRNAs in MVs, as compared to GBM cells for the presence of these elements. Surprisingly, among the enriched mRNAs set, the presence of both elements had more than twice the frequency as among the reduced mRNAs set (**Supplementary Table S3**). Since the previous zipcode studies on β-actin suggested the possible role of stem-loop structures,[18] we analyzed whether our 25-nt sequence predicts a stem-loop structure. The mFold web server (http://mfold.rna.albany.edu/?q=mfold) search predicted that this 25-nt putative zipcode sequence can assume a stem-loop configuration. Interestingly, the core "CTGCC" sequence and part of the miR-1289-binding sites are predicted to be located in this loop structure (**Supplementary Figure S2**). We next

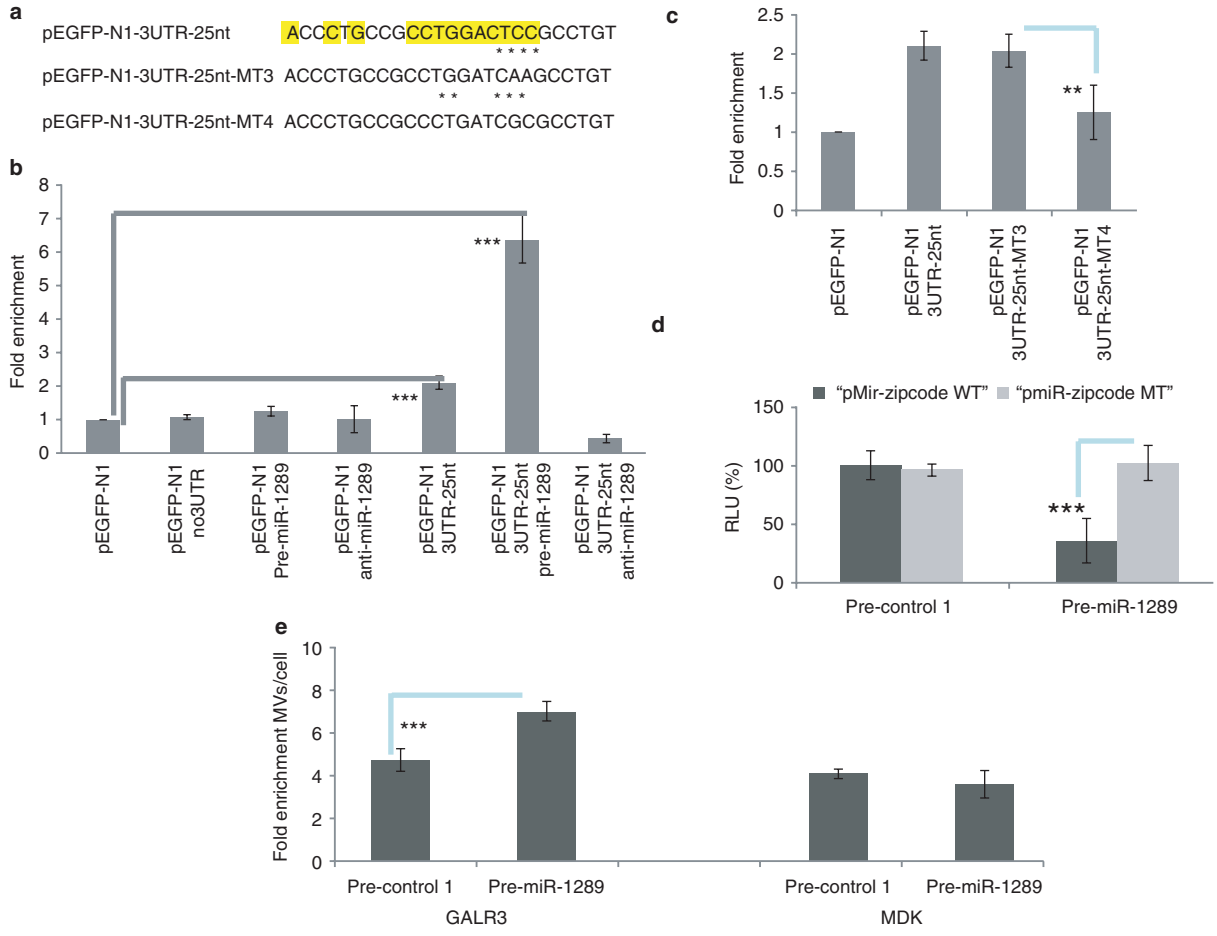

Figure 5 The effect of miRNA-1289 on EGFP mRNA enrichment within microvesicles (MVs). (**a**) miR-1289-binding site in yellow highlighted region (13 nt) and mutations in asterisks in the zipcode are shown for MT3 and MT4. (**b**) HEK-293T cells were transfected with indicated EGFP-expressing plasmids or co-transfected with plasmids and/or pre- or anti-miR-1289. Seventy-two hours later, total RNA was isolated from cells and MVs and quantitative reverse transcription (qRT)-PCR were performed for EGFP and GAPDH mRNAs. The data were normalized to the level of GAPDH mRNA in each sample and presented as fold enrichment in MVs. Microsomal EGFP mRNA enrichment is shown according to the EGFP/GAPDH ratios of pEGFP-N1, pEGFP-N1no 3′UTR, pEGFP-N1 + premiR-1289, pEGFP-N1 + anti-miR-1289, pEGFP-N1-3UTR-25nt, pEGFP-N1-3UTR-25nt + premiR-1289, pEGFP-N1-3UTR-25nt + anti-miR-1289. Student's *t*-test, $^{**}P < 0.01$. (**c**) Similar transfection and qRT-PCR were performed as in (**b**) including the mutated plasmids: pEGFP-N1-3UTR-25nt-MT3 and pEGFP-N1-3UTR-25nt-MT4. EGFP mRNA enrichment in MVs is shown using normalized values. (**d**) pMiR-report vectors containing the pMir-zipcode wild-type 25 nt or mt miR-1289 (MT4) binding sites in the 3′UTR were co-transfected into HEK-293T cells together with pre-miR-1289 or pre-control 1, as well as an expression cassette for Rluc. Two days later, Fluc activity in the cells was measured and normalized to Rluc activity. Five independent experiments were performed in triplicate and the values are expressed as mean ± SD. Student's *t*-test, $^{***}P < 0.001$. (**e**) GBM cells were transfected either with pre-miR-1289 or pre-control 1 and 72 hours later, MVs were collected as above and qRT-PCR was performed for GALR3, MDK, and GAPDH mRNAs. This experiment was performed in triplicate and the data were normalized to the level of GAPDH mRNA in each sample and presented as fold enrichment MVs/cell. The values are expressed as mean ± SD. Student's *t*-test, $^{***}P < 0.001$.

analyzed and compared the secondary structures of the four mutant sequences that we generated in this study using mFold (**Supplementary Figure S2**). In comparing the fold enrichment of the reporter mRNA in MVs:cells, it appears that the presence of both the "CTGCC" core sequence and part of the miR-1289 binding site on the loop are critical to sustain the twofold mRNA enrichment (**Supplementary Table S4**).

Discussion

MVs were first described almost three decades ago by Trams *et al.*[19] as exfoliated vesicles with ectoenzyme activity. In recent studies, they have proven valuable as a "transparent window" of biomarkers to monitor the disease status in patients, including cancer and neurodegenerative disorders.[20] Research in recent years has also pointed out their "cargo role" as a communication tool between cells in the horizontal transfer of RNAs and proteins between cells.[3,5,6,21] Despite these intensive studies, the molecular mechanism by which genetic materials are uploaded into and transferred by MVs is still unknown, although for shedding MVs RNA incorporation may have parallels with retrovirus budding from the plasma membrane.[22] In the present study, we have shown that there is a zipcode-like 25-nt sequence which contains a short "CTGCC" core domain on a stem-loop structure and carries

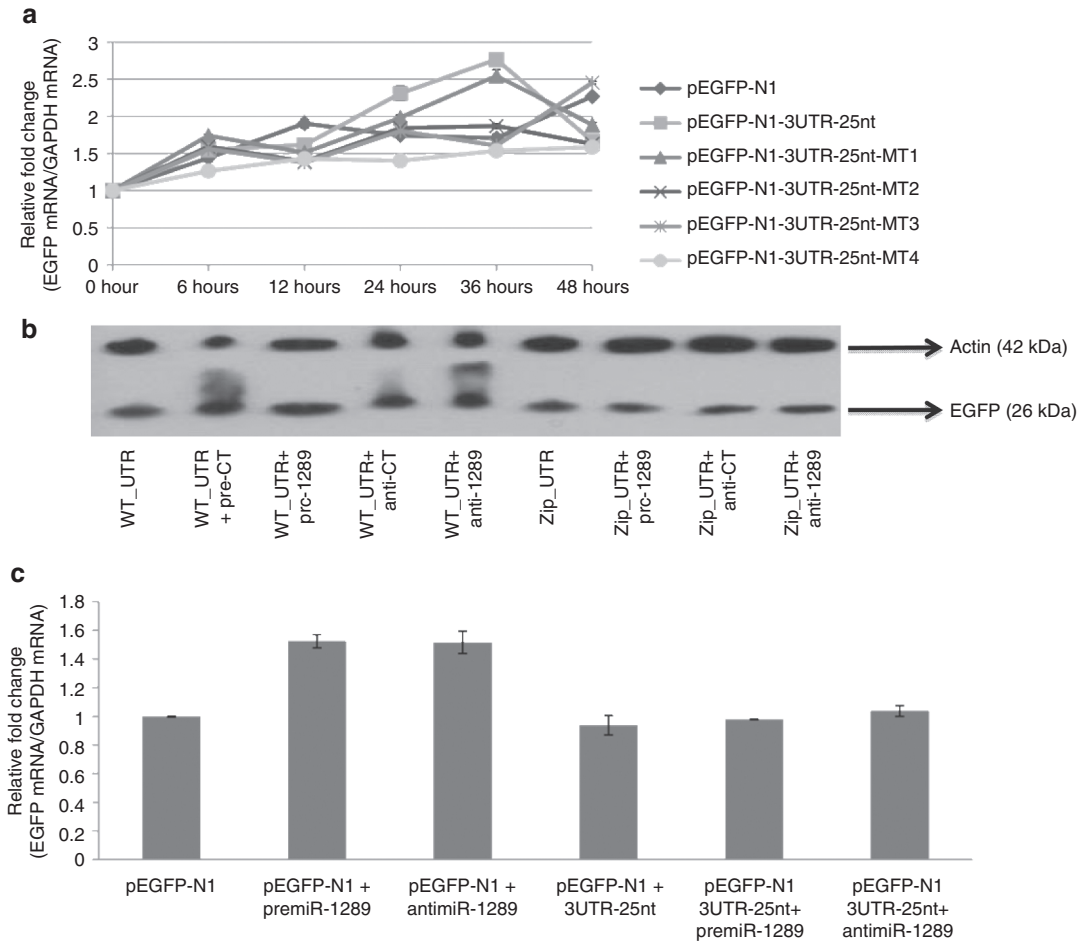

Figure 6 Effect of miR-1289 expression on the EGFP mRNA and protein levels. (**a**) HEK-293T cells were transfected overnight with pEGFP-N1 and other derived plasmids. Twelve hours later transfection medium was replaced (t = 0 hours). At different time points, total RNA was extracted and quantitative reverse transcription (qRT)-PCR was performed for EGFP and GAPDH mRNAs. EGFP mRNA levels were normalized to GAPDH mRNA. For each plasmid, EGFP mRNA level at t = 0 is taken as 1 and other levels at different time points are normalized accordingly. (**b**) HEK-293T cells were transfected with pEGFP-N1 or pEGFP-N1-3UTR-25nt alone or with pre-/anti-control (CT) or pre-/anti-miR-1289. Seventy-two hours after transfection cells were harvested and western blot analysis was performed with antibodies to EGFP and β-actin. (**c**) In parallel experiments, total RNA was isolated and qRT-PCR was performed using EGFP and GAPDH primers. EGFP mRNA levels were normalized to GAPDH mRNA.

a miR-1289 binding site in the 3UTRs of many of the most enriched mRNAs in MVs derived from human primary GBM cells and as well as melanoma cells. Furthermore, we have also shown that miR-1289 binds directly to this zipcode and orchestrates transfer mRNAs into MVs. This zipcode sequence can be used to increase the levels of mRNAs into MVs.

Studies focusing on intracellular mRNA localization started two decades ago and specific localization of mRNAs have been shown in various organisms and systems: budding yeast, *Drosophila*, *Xenopus*, and in mammalian cells including fibroblasts, oligodendrocytes, and neurons.[14,23,24] One type of these mRNA–protein complexes is found in cytoplasmic foci called processing bodies (P-bodies), which contain untranslated mRNAs and can serve as sites of mRNA degradation or storage.[25] Another study provided a link between miRNA function and mammalian P-bodies, as argonaute (AGO) proteins were found to be localized to mammalian P-bodies in a miRNA-dependent manner.[26] miRNAs typically associate with a complex of proteins that includes a member of the AGO family with which they form the RNA-induced silencing complex including target mRNAs.[27,28] In addition, Gibbings *et al*. showed a novel cellular mRNA storage site called GW-bodies containing GW182 and AGO2 proteins which is distinct from P-bodies and can serve as a novel storage depot for miRNA-mRNA loading.[29] These studies together with our observations suggested that not only P- or GW-bodies, but also MVs may be sites of miRNA–mRNA interaction involved in suppressing mRNA translation in the host cell. It remains to be investigated whether the zipcode-like sequence described in our study might also participate in mRNA transport into P- or GW-bodies. Another interesting question which will require further investigation is whether other members of the RNA-induced silencing complex machinery are involved in the mRNA enrichment in MVs mediated by a zipcode-like sequence.

One of the earliest studies on regulatory function of 3′UTRs in determining the cellular localization and translation of mRNAs came from analysis of chicken β-actin mRNA. A 54-nt sequence in the 3′UTR of β-actin mRNA was found

to be essential and sufficient for mRNA localization to the cell periphery.[30,31] This sequence also contains a hexanucleotide sequence (ACACCC), which is conserved and also forms a stem-loop structure in β-actin mRNA in other species.[18] This study also suggested that the functional protein–RNA interactions may depend on this stem-loop secondary structure. Interestingly, the zipcode-like sequence described in our study is also predicted to form a stem-loop secondary structure. Our mutation analysis showed that the core sequence of this zipcode, "CTGCC" is indispensable for increased transport of the reporter EGFP mRNAs into MVs and functions most efficiently within the context of the loop structure. Our findings suggest that the presence of both the CTGCC core sequence on the loop structure and the miR-1289 binding site in the 3′UTR have a critical role in increasing the incorporation of mRNAs into MVs.

Recent studies provided similarities between biogenesis of shed MVs and retrovirus budding, in particular, targeting signals such as oligomeric proteins with plasma membrane anchors can deliver proteins into MVs.[22,32] It remains to be investigated whether the zipcode-like sequence identified in the present study can act as a regulatory cis-element incorporated into the RNA-induced silencing complex with Ago 2 to direct some mRNAs into MVs or serves as a sorting signal or anchor to plasma membrane proteins involved in MV biogenesis.

Cancer MVs studies in recent years have raised interesting questions as to why tumor cells would seek to load particular mRNAs or miRNAs into MVs. Possible explanations are that mRNAs enrichment in MVs serves as "rubbish containers" whereby mRNAs are eliminated from the host cells through the miRNA-mRNA-RNA-induced silencing complex machinery, or that they serve as "storage containers" in which mRNAs are localized and transferred to other cells where they can be translated into proteins. The latter scenario seems to be very logical for tumors cells which express oncogenic mRNAs and proteins, such as for EGFRvIII in MVs which can be transferred to and active in recipient cells.[5–7] Perhaps, this storage mechanism is used to quickly initiate protein translation in recipient neighboring cells as a message of cancer transformation. Since MVs can also contain tumor suppressor miRNAs, it seems also possible that tumor cells may try to get rid of those miRNAs by transferring them in MVs. So, MV-mediated elimination or transfer of mRNAs and/or miRNAs could be used by tumor cells depending on the requirements of cellular conditions involved in tumor growth. In our study, based on the possible cancer-associated role(s) of the top four most enriched mRNAs in MVs released from GBM cells, it seems possible that cancer cells transfer these mRNAs to the other cells in which their translation might be promote tumorigenesis.

Among the four MV-enriched mRNAs within GBM vesicles are those corresponding to genes *MDK*, *COX8C*, *GALR3*, and *LOC653602*, the first three encode known cancer-associated proteins. One of these enriched mRNAs is the Galanin receptor 3 (GALR3). Berger *et al*.[33] has shown that both GAL and its specific receptors are elevated in human gliomas, as compared to normal tissue. Based on these findings, it seems highly likely that these receptors are cancer-related and transfer via MVs would support tumor growth through increasing an immortalized profile in surrounding stromal cells. This mRNA was increased in 50% in MVs derived from HEK-293 cells when the cells were transfected with pre-miR1289, consistent with our studies using the reporter zipcode mRNA.

Another of the most enriched mRNA in MVs found in our microarray was Midkine (MK or MDK), also known as neurite growth-promoting factor 2, which promotes cell proliferation, cell migration, and angiogenesis in several types of cancer in culture and *in vivo*.[34] The cancer-related activity of MK mRNA and protein expression are frequently elevated in many types of human carcinomas, including breast, lung, esophageal, stomach, colorectal, liver, ovary prostate, and urinary bladder carcinomas, as well as GBMs, neuroblastomas, and Wilms' tumors.[35–42] However, the MDK mRNA does not carry this zipcode and was not enriched in MVs derived from HEK-193 cells after transfection with pre-miR1289.

The 25-nt zipcode-like sequence elucidated in these studies corresponds to sequences within the 3′UTRs of many of the mRNAs enriched in MVs from GBM cells (**Figure 1**). Therefore, our sequence is not specific to one type of mRNA, but rather appears to represent a consensus sequence present in the 3′UTRs of a number of mRNAs enriched in tumor cell MVs. Our experimental analysis showed that this 25-nt zipcode-like sequence results in twofold mRNA enrichment in MVs, as compared to their cells of origin using a reporter mRNA. This enrichment was only seen in two cell types and may not be a universal mechanism. Our MV isolation strategy would result in our harvesting a variety of MV subtypes, including exosomes, shed MVs, and microparticles. Hence, it may be possible that the actual enrichment level in a subtype of MVs is much higher.

Discovery of a zipcode-like sequence which can target mRNAs to MVs is important in many different aspects of MV dynamics. MVs are now being considered as one of the essential intercellular communication tools and little is known about the basic biologic mechanisms underlying this form of communication. Understanding the physiological processes behind the transfer of RNA messages has broad ranging implications, from developmental studies to tumorigenesis, from cancer gene therapy to immunological studies.[11,12] An important aspect of this miRNA/mRNA transfer dynamic is that in some cases it may be a means of a cell eliminating and thereby decreasing translation of a specific protein, while in other cases it may serve to transmit miRNAs or mRNAs to recipient cells where they are active and modulate their phenotype. One potential approach for cancer gene therapy could be that this sequence can incorporated into the 3′UTR of therapeutic RNAs (including mRNAs, short hairpin RNAs, and noncoding regulatory RNAs) to enrich them in MVs, which, in turn, can serve as vehicles to deliver them to cells *in vivo* through on-site donor cells or through injection of loaded MVs.

Materials and Methods

Cell culture. HEK-293T cells (obtained from Dr Maria Calos, Stanford University, Stanford, CA) were cultured in Dulbecco's modified Eagle's medium (Cellgro; Mediatech, Manassas, VA)

containing 10% fetal bovine serum, 100 IU/ml penicillin. and 100 µg/ml streptomycin. Cells are incubated at 37 °C in a 5% CO_2 atmosphere. Cells were determined to be mycoplasma negative by testing with a mycoplasma detection kit (MycoAlert, Mycoplasma Detection Assay; Lonza, Rockland, ME).

Transfection of HEK-293T cells and MV isolation. Cultures of HEK-293T cells (75–80% confluent) were transfected with 7 µg pEGFP-N1 original or derived plasmids per each 200-mm plate with Lipofectamine 2000 (Invitrogen, Carlsbad, CA; 11668-019), according to manufacturer's protocol. Six hours later, transfection media was removed and replaced with Dulbecco's modified Eagle's medium containing 5% MV-free fetal bovine serum. MV-free fetal bovine serum is obtained after the ultracentrifugation and filtration process.[5] Three days after transfection, MVs were isolated from a total of 39-ml cell media from two plates through serial centrifugation: initial centrifugation was done at 300*g* for 15 minutes followed by 16,000*g* for 30 minutes. Then, the supernatant was filtered through 0.22-µm filters (Millex; Millipore, Billerica, MA) into Beckman Quick seal tubes. Finally, ultracentrifugation was performed at 110,000*g* for 90 minutes using a 70Ti rotor (Beckman Coulter, Brea, CA). MVs were resuspended in 50 µl twice-filtered 1× phosphate-buffered saline.

miRNA overexpression and inhibition. Precursory and inhibitory miRNAs used for our experiments were as follows: pre-miR-1289 (Ambion, Foster City, CA; AM17100), pre-control 1 (AM17110), anti-miRNA-1289 (AM17000), and anti-miR miRNA inhibitors—negative control 1 (AM17010). Both pre-miR and anti-miRs were used at 50 nmol/l final concentration for co-transfection experiments.

Total RNA isolation, reverse transcription, and real-time qPCR. We isolated total RNA from both cell pellets and MVs using a miRvana isolation kit (Ambion), according to manufacturer's protocol. Upon elution of RNA, it was treated with 1 µl 2U *DNase*I (Ambion) in 30 µl of total reaction for 30 minutes at 37 °C to eliminate any residual DNA. Following the RNA isolation, complementary DNA was generated using Omniscript RT kit-50 (Qiagen, Valencia, CA) using 100 ng of total RNA from MVs or 1 µg of total RNA from cell pellets, according to manufacturer's recommendations. mRNA levels were quantified with Applied Biosystems 7000 series quantitative-PCR. GAPDH mRNA was used for normalization purposes. The primers used in this study were as follows: GALR3-forward: 5′-CATGTACGCCAGCAGCTTTA, GALR3-reverse: 5′-ACGGTGCCGTAGTAGCTGAG; MDK-forward: 5′-CGGTCGCCAAAAAGAAAGAT,MDK-reverse:5′-GGCTCC AAACTCCTTCTTCC GAPDH-forward: 5′-GAAGGTGAAGG TCGGAGT, GAPDH-reverse: 5′-GAAGATGGTGATGGGA TTTC, and EGFP-forward: 5′-CCTGAAGTTCATCTGCACCA, and EGFP-reverse: 5′-GGTCTTGTAGTTGCCGTCGT.

Molecular cloning and site-specific mutagenesis. For microsomal enrichment assays, we used pEGFP-N1 (Clontech) as the parental vector. The 3′UTR of EGFP was replaced with the putative 25-nt zipcode at the Afl-2 and Not-1 sites and mutants of this sequence were inserted at the same sites (**Supplementary Figure S4**). In order to maintain mRNA stability, part of the 3′UTR of the original construct was maintained to retain the SV40 polyA addition site. The 3′UTRs of pEGFP-N1-3UTR-25nt, pEGFP-N1-NO3UTR, and pEGFP-N1-3UTR-25nt-MT4 inserts were generated by oligonucleotide annealing, as described.[43] These oligonucleotide sequences as follows: pEGFP-N1-3UTR-25nt (5′-GGCCGCACCCTGC-CGCCTGGACTCCGCCTGTACAAATAAAGCAATAGCAT-CACAAATTTCACAAATAAAGCATTTTTTTCACTGCC-3′), pEGFP-N1-NO3UTR (5′-GGCCGCACAAATAAAGCAATAG-CATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCC-3′), and pEGFP-N1-3UTR-25nt-MT4 (5′-GGCCGCACCCTGC-CGCCCTGATCGCGCCTGTACAAATAAAGCAATAGCAT-CACAAATTTCACAAATAAAGCATTTTTTTCACTGCC-3′). pEGFP-N1-3UTR-25nt-MT1, pEGFP-N1-3UTR-25nt-MT2 and pEGFP-N1-3UTR-25nt-MT3 plasmids were derived from pEGFP-N1-3UTR-25nt using Quickchange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). Primer sequences used in site directed mutagenesis reactions as follows: MT1 forward: 5′-CAAGTAAAGCGGCCGCACCGCATGGC-CTGGACTCCGCCTGTACMT1 reverse: 5′-GTACAGGCG-GAGTCCAGGCCATGCGGTGCGGCCGCTTTACTTG MT2 forward: 5′-CAAGTAAAGCGGCCGCACCACTTAGCCT-GGACTCCGCCTGTAC MT2 reverse: 5′ GTACAGGCG-GAGTCCAGGCTAAGTGGTGCGGCCGCTTTACTTG MT3 forward: 5′-CCGCACCCTGCCGCCTGGATCAAGCCTGTA-CAAATAAAGCAATAGC MT3 reverse: 5′-GCTATTGCTTT-ATTTGTACAGGCTTGATCCAGGCGGCAGGGTGCGG.

Western blots. Three days after transfection, HEK-293T cells were rinsed with 1× phosphate-buffered saline and harvested, and total proteins were separated by pre-cast NuPAGE 4–12% Bis–Tris (Invitrogen) polyacrylamide gel electrophoresis. Then proteins were transferred onto nitrocellulose membranes; membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.05% Tween-20[44] overnight at 4 °C. The antibodies used were: monoclonal EGFP (1:5,000; Invitrogen; 33-2600) and β-actin (1:5,000; Sigma, St Louis, MO; A5356). Bands were visualized using the ECL system according to instructions provided by the supplier (Amersham, Buckinghamshire, UK).

Luciferase miRNA target reporter assay. For the validation of the miR-1289 binding site in the putative MV zipcode, we used the pMir-Report system (Ambion, AM5795), as described.[45] In this system, the 3′UTR of firefly luciferase (Fluc) gene was replaced either with the putative MV zipcode or the MT1289 mutated version of this zipcode. DNA oligonucleotides were synthesized as sense and antisense templates of the 25-nt sequence and the mutant sequence. Then, they were annealed and cloned into pMir-Report between the *Hind*III and *Spe*I sites. The oligonucleotides used in these studies were as follows: 25-nt zipcode-UTR (5′-CTAGTACCCT-GCCGCCTGGACTCCGCCTGTA-3′) 25-nt zipcode-MT4: (5′-CTAGTACCCTGCCGCCCTGATCGCGCCTGTA-3′).

HEK-293T cells are co-transfected with pMir-Report vectors and either pre-miR-1289 or pre-miR negative control 1. Two days after transfection, cells were lysed and the luciferase activity was measured. Another plasmid with a *Renilla* luciferase (Rluc) expression cassette was co-transfected and used for normalization.[45]

Multiple sequence alignment and zipcode scanning. The list of 50 most enriched and most reduced mRNAs in MVs as compared to GBM cells were generated from microarray data of Skog *et al*.[5] The 3′UTR sequences of the top enriched 20 genes were aligned using the multiple sequence alignment tool ClustalW (Clustal W2-http://www.clustal.org/) under the following conditions: fast alignment method, gap open 10, gap extend 0.2, and DNA weight matrix ClustalW. In addition, for deep alignment we used a slow alignment method. In order to eliminate false negative hits, we excluded polyA sequences in the 3′ ends of the sequences. Sequence similarities were found through pairwise alignment option of the BLAST (BLAST-http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide blast (blastn) program was used with minimum hit length of 7 nt.

miRNA-binding site predictions. miRNA targeting sequences within the 25-nt putative MV zipcode were checked using miRBase (http://www.mirbase.org/). Predicted target transcripts of miR-1289 were collected and combined from three different miRNA databases: TargetScanHuman (http://www.targetscan.org/), miRNA.org, and miRWalk (www.ma.uni-heidelberg.de). In addition, blastn was used to detect additional similarities which were 7 base pairs or longer.

The microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo) with GEO series accession number GSE35444.

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Supplementary material

Figure S1. Secondary structure of the 25 nt zipcode.
Figure S2. Secondary structure of the zipcode-mutated sequences.
Figure S3. EGFP mRNAs bearing zipcode 3′UTR are stable and able to be transported into MVs.
Figure S4. 3′UTR sequences of the plasmids that were used.
Table S1. The list of top 20 mRNAs enriched in MVs.
Table S2. Alignment of the 3′UTR of mRNAs with potential miR-1289-binding sites.
Table S3. Ratio of mRNAs with core sequence and/or miR-1289-binding site.
Table S4. Relative fold enrichments of the zipcode and its mutated version in MVs.

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Supplementary Information accompanies this paper on the Molecular Therapy–Nucleic Acids website (http://www.nature.com/mtna)